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<b>(54) Title:</b> METHODS OF SUPPRESSING AUTOIMMUNE RESPONSE  <b>(57) Abstract</b>  Tissue cells of an animal transformed with a recombinant vector construct that (a) expresses a protein or active portion thereof; (b) transcribes an antisense message; or (c) transcribes a ribozyme capable of inhibiting MHC antigen presentation, for use in suppressing an autoimmune response are provided. In a related aspect, the cells are transformed with two or more proteins, antisense or ribozymes, or combinations thereof.		

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## Description

### **METHODS OF SUPPRESSING AUTOIMMUNE RESPONSE**

5

#### Technical Field

The present invention relates generally to the field of autoimmunity, and more specifically, to methods of suppressing T-cell recognition of host tissues.

#### 10 Background of the Invention

Autoimmunity refers to the reaction of the immune system against the body's own tissues, and is characterized by either the production of antibodies or immune effector T-cells that react with host tissue. Several diseases attributed to autoimmunity include multiple sclerosis (MS), rheumatoid arthritis, diabetes and uveitis.

15 MS is a neurological disorder characterized by recurrent incidence of axon demyelination in the optic nerve, brain, and spinal cord. Although it affects approximately one million people worldwide, the incidence is higher in populations living above the 37th parallel. This progressive disease may be linked to a defect in the immune system which may cause the self-destruction of myelin sheaths in both the  
20 central and peripheral nervous systems. MS patients are genetically susceptible to the onset of this disease following infection by a virus containing amino acid sequences similar to normal myelin. There is a strong association between MS and the human leukocyte antigen (HLA) HLA-DR2, providing support for this genetic predisposition. These patients have lymphocytes that mistakenly identify host myelin, resulting in an  
25 autoimmune response.

MS affects persons under 55 years of age primarily of Western European lineage. Common symptoms include partial loss of vision and problems with speech, balance, and general motor coordination. Symptoms may cease after a few days or weeks but can reoccur after months or years. Eventually relapses lead to increasing  
30 disability and weakness. In some instances the symptoms are steadily progressive from their onset and disability develops at a relatively early stage.

Although no known treatment exists to prevent progression of the disorder, corticosteroids, such as prednisone, can hasten recovery from relapse. However, the resulting damage remains unchanged. Intensive immunosuppressive  
35 therapy with cyclophosphamide or azathioprine may aid in arresting the course of chronic progressive active MS, but the possibility of opportunistic infection increases.

One of the symptoms, spasticity, is treated with the drug Danthrolene, which weakens muscle contraction by interfering with the role of calcium. Although this leads to a decrease in the spasm intensity, treatment cannot be administered to patients with poor respiratory function or myocardial disease. Another drug, Baclofen, has been the most effective in treating spasticity of spinal origin. Unfortunately, associated side effects include gastrointestinal disturbances, lassitude, fatigue, sedation, unsteadiness, confusion and hallucinations. While still another drug, Diazepam, modifies spasticity, effective dosages often cause intolerable drowsiness. Alternatively, intrathecal injection of phenol or absolute alcohol has been used to reduce spasticity selectively in one or more important muscles.

Rheumatoid arthritis is an autoimmune disease characterized by chronic systemic inflammation specifically affecting synovial membranes of the joints. Viral infection in a genetically susceptible individual leads to either a cross reaction between antiviral antibodies and joint tissue antigen, or deviation of lymphocyte function by incorporation of viral DNA into the cell genome. The pathology of the disease includes chronic synovitis and pannus formation. Synovitis refers to the excessive inflammation of the synovial membranes surrounding the joints, thereby causing severe pain, while pannus eventually results in fibroid alkalosis by eroding cartilage, bone, ligaments, and tendons. The prevalence of rheumatoid arthritis in the general population is 1%-2%, with female patients outnumbering males 3:1. In general, the age at onset ranges from 20-40 years, although this disease may begin at any age.

The primary objectives for treating rheumatoid arthritis are reduction of inflammation and pain, preservation of function, and prevention of deformity. Several treatments have been used to accomplish these goals including non-steroidal anti-inflammatory drugs, anti-malarial drugs, gold salts, corticosteroids, methotrexate, azathioprine and penicillamine. Nonsteroidal anti-inflammatory drugs include aspirin, ibuprofen, fenoprofen, naproxen, tolmetin, flurbiprofen, sulindac, meclofenamate sodium, piroxicam, diclofenac, and ketoprofen. However, these drugs induce gastric ulceration, perforation, or aggravating inflammatory bowel disease, and in some cases result in renal toxicity.

The anti-malaria drug, Plaquenil (hydroxychloroquine sulfate), is used in patients with mild cases of rheumatoid arthritis because of its low toxicity as compared to nonsteroidal anti-inflammatory drugs. However, only 25% of those treated respond to this drug and in some cases only after 3-6 months of therapy. Side effects include pigmentary retinitis, neuropathologies, and myopathologies of both skeletal and cardiac muscle.

In some patients, gold salts have been shown to retard bone erosions associated with rheumatoid arthritis. Thirty-two percent of these patients experience toxic side effects similar to heavy metal poisoning. The toxicity manifests itself as dermatitis, stomatitis, neutropenia, nephritis and nitritoid reactions.

5 Corticosteroids are generally used for their immediate anti-inflammatory effect without altering the natural progression of rheumatoid arthritis. Unfortunately, they mask the underlying disease, and therefore increase the tendency of the patient and physician to neglect general supportive treatment, physical therapy and orthopedic measures.

10 Methotrexate is given to patients with severe rheumatoid arthritis who fail to respond to nonsteroidal anti-inflammatory drugs and gold salts. This drug produces beneficial effects within two to four weeks as compared to gold salts which take two to six months. However, associated disadvantages include gastric irritation, stomatitis, pneumonitis, fibrosis, and cirrhosis. Therefore, liver biopsies are performed  
15 periodically to monitor treated individuals.

Azathioprine, like methotrexate, is effective for the treatment of severe rheumatoid arthritis. However, its use is restricted because of the potential for severe toxicity, including leukopenia, thrombocytopenia, and immunosuppression complicated by opportunistic infection.

20 Penicillamine is also effective for treating severe rheumatoid arthritis. However, up to one-half of the patients experience some side effects, such as oral ulcers, loss of taste, fever, rash, thrombocytopenia, leukopenia and aplastic anemia. Other immune complex diseases appear to be induced by the drug including myasthenia gravis, systemic lupus erythematosus, polymyositis, and Goodpasture's syndrome.

25 Diabetes mellitus is a disease characterized by an absence of circulating insulin, elevated plasma glucagon, and destruction of pancreatic B cells resulting in a disordered metabolism and hyperglycemia. The disease is most often treated by insulin injection, which prevents ketosis, reduces hyperglucagonemia, and decreases the elevated blood glucose level. Two major classifications of this disease are type I or  
30 insulin-dependent diabetes mellitus (IDDM) and type II or non-insulin-dependent diabetes mellitus (NIDDM). IDDM, occurs most commonly in children. Twenty percent of all diabetics in Scandinavia suffer from IDDM decreasing to 13% in southern Europe, 8% in the United States, and less than 1% in Japan and China.

Certain HLA have been associated with IDDM. HLA-DR3 and HLA-  
35 DR4 are present in 95% of patients as compared to 45%-50% in controls. In addition, antigen HLA-DQw3.2 is present in DR4 patients with IDDM, while the protective gene

HLA-DQ3.1 is found predominantly in DR4 controls. Anti-insulin and circulating islet cell antibodies have been detected in 85% of patients in the first few weeks following the onset of diabetes. Considering these immune characteristics, IDDM is believed to result from an infectious or toxic environmental insult to pancreatic B cells of individuals whose immune system is genetically predisposed to develop an autoimmune response against altered pancreatic B cell antigens. Factors that effect B cell function include damage caused by viruses such as mumps or coxsackie B4 virus, toxic chemical agents or cytotoxins, and antibodies released from sensitized immunocytes. Sustained hyperglycemia causes osmotic diuresis, resulting in increased urination. This lowered plasma volume produces dizziness and weakness that is further exacerbated by potassium loss and catabolism of muscle protein. Acute stages of ketoacidosis exacerbates the dehydration and hyperosmolarity by producing anorexia, nausea and vomiting. This condition interferes with oral fluid replacement and as serum osmolarity increases, the patient experiences impaired consciousness eventually progressing to coma.

Type II diabetes occurs predominantly in adults. NIDDM is not associated with HLA markers, is non-ketotic, lacks islet cell antibodies, and does not require insulin therapy to sustain life. Although the cause of this form of diabetes is unclear, an unknown primary genetic factor is implicated which is aggravated by enhancers of insulin resistance such as aging and abdominal visceral obesity. Genetic influences have been further supported by epidemiological data showing that when one monozygotic twin over forty years of age develops diabetes, the second will develop the disease within the same year.

In the United States over 90% of all diabetics are type II. These patients are initially asymptomatic, although chronic skin infections are common. An estimated 7 million people in the United States suffer from diabetes of which 560,000 have type I diabetes. Treatment generally requires controlling dietary caloric intake to maintain weight. Other treatments involve the use of hypoglycemic drugs such as sulfonylureas. Due to the limited duration in the system, these drugs must be continuously administered to insulinopenic diabetics to improve insulin release.

Drugs from the biguanides class were introduced in the 1950's but were discontinued in the United States because of their implication in lactic acidosis. Metformin has been used in France since 1957 and is currently awaiting FDA approval. This drug is used in conjunction with sulfonylureas and has many side effects including anorexia, nausea, vomiting, abdominal discomfort and diarrhea.

Insulin injection is used for type I and non-obese type II diabetes. However, subcutaneous injections cannot reproduce physiological patterns of intraportal insulin secretions. Therefore, treatment requires constant monitoring of blood glucose levels followed by insulin injections over the patient's lifetime.

5 Uveitis is an intraocular inflammatory disease present in the anterior or posterior segment of the eye or equally distributed between the two. It is categorized as acute or chronic and granulomatous or nongranulomatous.

Anterior uveitis is characterized by inflammatory cells within the aqueous humor. In granulomatous anterior uveitis, large precipitates and iris nodules cause blurred vision and inflammation. Diseases producing granulomatous anterior uveitis include sarcoidosis, tuberculosis, syphilis, toxoplasmosis, Vogt-Koyanagi-Harada syndrome and sympathetic ophthalmia.

In non-granulomatous anterior uveitis, the precipitates are smaller and lack iris nodules. This causes unilateral pain, redness, photophobia and loss of vision. In severe non-granulomatous anterior uveitis, fibrin is present within the anterior chamber. Systemic disorders associated with acute non-granulomatous anterior uveitis are the HLA-B27-related conditions sacroilitis, ankyloing spondylitis, Reiter's syndrome, psoriasis, ulcerative colitis and Chron's disease. Other infections that may cause non-granulomatous anterior uvieitis are herpes simplex and herpes zoster.

20 In posterior uveitis, there are cells in the vitreous humor and inflammatory lesions in the retina or choroid. Visual loss may be due to vitreous haze, opacities, inflammatory lesions involving the macula, macular edema, retinal vein occlusion or associated optic neuropathy. Autoimmune retinal vasculitis and pars planitis are conditions that produce posterior uveitis.

25 There are limited treatments for uveitis. Anterior uveitis may be treated with topical corticosteroids, while posterior uveitis requires systemic corticosteroid therapy. Posterior uveitis may also be treated through systemic immunosuppression with azathioprine or cyclosporine. However, high dosage use can result in hepatotoxicity and nephrotoxicity. Other side effects include renal dysfunction, tremor, 30 hirsutism, hypertension and gum hyperplasia.

Consequently, there is a need in the art for improved methods of suppressing the autoimmune response without the side effects or disadvantages associated with previously described methods. The present invention fulfills these needs and further provides other related advantages.

### Summary of the Invention

The present invention provides methods for inhibiting MHC antigen presentation in order to suppress T-cell recognition of host tissues. Within one aspect of the present invention, methods are provided for suppressing the autoimmune response  
5 within an animal, comprising transforming tissue cells of an animal with a recombinant vector construct that expresses a protein or an active portion of a protein capable of inhibiting MHC antigen presentation, such that an autoimmune response against the cells is suppressed. Within one embodiment of the present invention, the recombinant vector construct directs the expression of a protein capable of binding  $\beta_2$ -microglobulin, such  
10 as H301. In another embodiment, the recombinant vector construct directs the expression of a protein capable of binding the MHC class I heavy chain molecule intracellularly, such as E3/19K.

Within another aspect of the invention, a method is provided for suppressing an autoimmune response within an animal, comprising transforming tissue  
15 cells of an animal with a recombinant vector construct that transcribes an antisense message capable of inhibiting MHC antigen presentation, such that an autoimmune response against the cells is suppressed. Within various embodiments of the present invention, the recombinant vector construct transcribes an antisense message which binds a conserved region of MHC class I heavy chain transcripts,  $\beta_2$ -microglobulin  
20 transcript, or the PSF1 transporter protein transcript.

Within still another aspect of the invention, a method is provided for suppressing an autoimmune response within an animal, comprising transforming tissue cells of an animal with a recombinant vector construct that transcribes a ribozyme capable of inhibiting MHC antigen presentation, such that an autoimmune response  
25 against the cells is suppressed. Within various embodiments of the present invention, the recombinant vector construct transcribes a ribozyme that cleaves a conserved region of MHC class I heavy chain transcripts,  $\beta_2$ -microglobulin transcript, or the PSF1 transporter protein transcript.

Within yet another aspect of the invention, a method is provided for  
30 suppressing an autoimmune response within an animal, comprising transforming tissue cells of an animal with a multivalent recombinant vector construct that expresses a protein or active portion of a protein capable of inhibiting MHC antigen presentation, and an antisense message or ribozyme capable of inhibiting MHC antigen presentation, such that an autoimmune response against the cells is suppressed. Within a related  
35 aspect of the invention, the multivalent recombinant vector construct directs the expression of an antisense message and a ribozyme capable of inhibiting MHC antigen



presentation, such that an autoimmune response to the cells is suppressed. Within another related aspect of the present invention, the multivalent recombinant vector construct directs the expression of two or more proteins or active portions of proteins, two or more antisense messages, or two or more ribozymes capable of inhibiting MHC antigen presentation, such that an autoimmune response to the cells is suppressed.

Within various embodiments of the invention, the multivalent recombinant vector construct expresses or transcribes at least two of the following in any combination: a protein or active portion of the proteins E3/19K or H301, an antisense message that binds the transcript of a conserved region of MHC class I heavy chains,  $\beta_2$ -microglobulin or PSF1 transporter protein, or a ribozyme that cleaves the transcript of a conserved region of the MHC class I heavy chains,  $\beta_2$ -microglobulin or PSF1 transporter protein.

Within preferred embodiments, the recombinant vector construct is a recombinant viral vector construct. Within a particularly preferred embodiment, the recombinant vector construct is a recombinant retroviral vector construct. Within other embodiments, the recombinant vector construct is carried by a virus selected from the group consisting of togaviridae, picornaviridae, poxviridae, adenoviridae, parvoviridae, herpesviridae, paramyxoviridae and coronaviridae.

Within the methods briefly discussed above, suitable tissue cells of an animal include myelin nerve sheath cells, synovial membrane cells, pancreatic islet cells, hepatocytes and keratocytes. Within a preferred embodiment of the present invention, the animal cells are transformed *in vivo* by direct injection of a recombinant vector construct.

These and other aspects of the present invention will become evident upon reference to the following detailed description.

#### Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

"Autoimmune response" as used herein refers to a condition characterized by a specific humoral or cell-mediated immune response against constituents of the body's own tissue. Within the context of the present invention, "suppression" of the autoimmune response refers to interference with MHC antigen presentation, such that an immune response is diminished or prevented.

"Transforming" tissue cells refers to the transduction or transfection of tissue cells by any of a variety of means recognized by those skilled in the art, such that

the transformed tissue cell expresses additional polynucleotides as compared to a tissue cell prior to the transforming event.

"Recombinant vector construct" or "vector construct" refers to an assembly which is capable of expressing sequences or genes of interest. The vector  
5 construct must include promoter elements and may include a signal that directs polyadenylation. In addition, the vector construct preferably includes a sequence which, when transcribed, is operably linked to the sequences or genes of interest and acts as a translation initiation sequence. Preferably, the vector construct includes a selectable  
10 marker such as neomycin, thymidine kinase, hygromycin, phleomycin, histidinol, or dihydrofolate reductase (DHFR), as well as one or more restriction sites and a translation termination sequence. In addition, if the vector construct is used to make a retroviral particle, the vector construct must include a retroviral packaging signal and LTRs appropriate to the retrovirus used, provided these are not already present. The  
15 vector construct can also be used in combination with other viral vectors or inserted physically into cells or tissues as described below. As noted above, the vector construct includes a sequence that encodes a protein or active portion of the protein, antisense message or ribozyme. Such sequences are designed to inhibit MHC antigen presentation, in order to suppress an autoimmune response of class I restricted T-cells against transformed tissues cells.

20 In general, the recombinant vector constructs described herein are prepared by selecting a plasmid with a strong promoter, and appropriate restriction sites for insertion of DNA sequences of interest downstream from the promoter. As noted above, the vector construct may have a gene encoding antibiotic resistance for selection as well as termination and polyadenylation signals. Additional elements may include  
25 enhancers and introns with functional splice donor and acceptor sites.

The construction of multivalent recombinant vector constructs may require two promoters when two proteins are being expressed, because one promoter may not ensure adequate levels of gene expression of the second gene. In particular, where the vector construct expresses an antisense message or ribozyme, a second  
30 promoter may not be necessary. Within certain embodiments, an internal ribosome binding site (IRBS) or herpes simplex virus thymidine kinase (HSVTK) promoter is placed in conjunction with the second gene of interest in order to boost the levels of gene expression of the second gene. Briefly, with respect to IRBS, the upstream untranslated region of the immunoglobulin heavy chain binding protein has been shown  
35 to support the internal engagement of a bicistronic message (Jacejak et al., Nature 353:90, 1991). This sequence is small, approximately 300 base pairs, and may readily be

incorporated into a vector in order to express multiple genes from a multi-cistronic message whose cistrons begin with this sequence.

Where the recombinant vector construct is carried by a virus, such constructs are prepared by inserting sequences of a virus containing the promoter, splicing, and polyadenylation signals into plasmids containing the desired gene of interest using methods well known in the art. The recombinant viral vector containing the gene of interest can replicate to high copy number after transduction into the target tissue cells.

Subsequent to preparation of the recombinant vector construct, it may be preferable to assess the ability of vector transformed cells to down regulate MHC presentation. In general, such assessments may be performed by Western blot, FACS analysis, or by other methods recognized by those skilled in the art.

Within preferred embodiments, the recombinant vector construct is carried by a retrovirus. Retroviruses are RNA viruses with a single positive strand genome which in general, are nonlytic. Upon infection, the retrovirus reverse transcribes its RNA into DNA, forming a provirus which is inserted into the host cell genome. Preparation of retroviral constructs for use in the present invention is described in greater detail in an application entitled "Recombinant Retroviruses" (U.S.S.N. 07/586,603, filed September 21, 1990) herein incorporated by reference. The retroviral genome can be divided conceptually into two parts. The "trans-acting" portion consists of the region coding for viral structural proteins, including the group specific antigen (gag) gene for synthesis of the core coat proteins; the pol gene for the synthesis of the reverse transcriptase and integrase enzymes; and the envelope (env) gene for the synthesis of envelope glycoproteins. The "cis-acting" portion consists of regions of the genome that is finally packaged into the viral particle. These regions include the packaging signal, long terminal repeats (LTR) with promoters and polyadenylation sites, and two start sites for DNA replication. The internal or "trans-acting" part of the cloned provirus is replaced by the gene of interest to create a "vector construct". When the vector construct is placed into a cell where viral packaging proteins are present (see U.S.S.N. 07/800,921), the transcribed RNA will be packaged as a viral particle which, in turn, will bud off from the cell. These particles are used to transduce tissue cells, allowing the vector construct to integrate into the cell genome. Although the vector construct express its gene product, the virus carrying it is replication defective because the trans-acting portion of the viral genome is absent. Various assays may be utilized in order to detect the presence of any replication competent infectious retrovirus. One preferred assay is the extended S<sup>+</sup>L<sup>-</sup> assay described in Example 9. Preferred retroviral

vectors are murine leukemia amphotropic or xenotropic or VsVg pseudotype vectors (see WO 92/14829; incorporated herein by reference).

Recombinant vector constructs may also be developed and utilized with a variety of viral carriers including, for example, poliovirus (Evans et al., Nature 339:385, 1989, and Sabin et al., J. of Biol. Standardization 1:115, 1973) (ATCC VR-58); rhinovirus (Arnold et al., J. Cell. Biochem. L401, 1990) (ATCC VR-1110); pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., PNAS 86:317, 1989; Flexner et al., Ann. N.Y. Acad. Sci. 569:86, 1989; Flexner et al., Vaccine 8:17, 1990; U.S. 4,603,112 and U.S. 4,769,330; WO 89/01973) (ATCC VR-111; ATCC VR-2010); SV40 (Mulligan et al., Nature 277:108, 1979) (ATCC VR-305), (Madzak et al., J. Gen. Vir. 73:1533, 1992); influenza virus (Luytjes et al., Cell 59:1107, 1989; McMichael et al., The New England Journal of Medicine 309:13, 1983; and Yap et al., Nature 273:238, 1978) (ATCC VR-797); adenovirus (Berkner, et al., Biotechniques 6:616, 1988, and Rosenfeld et al., Science 252:431, 1991) (ATCC VR-1); parvovirus such as adeno-associated virus (Samulski et al., J. Vir. 63:3822, 1989, and Mendelson et al., Virology 166:154, 1988) (ATCC VR-645); herpes simplex virus (Kit et al., Adv. Exp. Med. Biol. 215:219, 1989) (ATCC VR-977; ATCC VR-260); HIV (EPO 386,882, Buchschacher et al., J. Vir. 66:2731, 1992); measles virus (EPO 440,219) (ATCC VR-24); Sindbis virus (Xiong et al., Science 234:1188, 1989) (ATCC VR-68); and coronavirus (Hamre et al., Proc. Soc. Exp. Biol. Med. 121:190, 1966) (ATCC VR-740). It will be evident to those in the art that the viral carriers noted above may need to be modified to express proteins, antisense messages or ribozymes capable of inhibiting MHC antigen presentation.

Once a vector construct has been prepared, it may be administered to a warm-blooded animal in order to transform tissue cells through a variety of routes, including *in vivo* by direct injection. More specifically, naked DNA or a recombinant vector construct containing a sequence that codes for a protein or active portion of a protein, an antisense message or ribozyme sequence capable of inhibiting MHC antigen presentation, can be directly injected into the interstitial space of tissues including muscle, brain, liver, skin, synovial membrane cells, pancreatic islet cells, and keratocytes (see WO 90/11092). Other representative examples of *in vivo* administration of vector constructs include transfection by various physical methods, such as lipofection (Felgner et al., PNAS 84:7413, 1989); microprojectile bombardment (Williams et al., PNAS 88:2726, 1991); liposomes (Wang et al., PNAS 84:7851, 1987); calcium phosphate (Dubensky et al., PNAS 81:7529, 1984); DNA ligand complexes (Wu et al., J. of Biol. Chem. 264:16985, 1989; Cotten et al., PNAS 89:6094, 1992). As noted above, the

vector construct may be carried by a virus such as vaccinia, Sindbis, or corona. Further, methods for administering a vector construct via a retroviral vector by direct injection are described in greater detail in an application entitled "Recombinant Retroviruses" (U.S.S.N. 07/586,603) herein incorporated by reference.

5 As discussed above, the present invention provides methods and compositions suitable for inhibiting MHC antigen presentation in order to suppress the autoimmune response of the host. Briefly, CTL are specifically activated by the display of peptides in the context of self MHC molecules along with accessory molecules such as CD8, intercellular adhesion molecule -1 (ICAM-1), ICAM-2, (Singer, Science 255:  
10 1671, 1992; RAO, Crit.Rev.Immunol. 10: 495, 1991 leukocyte functional antigen-1 (LFA-1) (Altmann et al., Nature 338:521, 1989), the B7/BB1 molecule (Freeman et al., J. Immunol. 143: 2714, 1989), LFA-3, or other cell adhesion molecules. Antigenic peptide presentation in association with MHC class I molecules leads to CTL activation. Transfer and stable integration of specific sequences capable of expressing products  
15 expected to inhibit MHC antigen presentation block activation of T-cells, such as CD8<sup>+</sup> CTL, and therefore suppress the autoimmune response. A standard CTL assay is used to detect this response, as described in detail in Example 13. Components of the antigen presentation pathway include the 45Kd MHC class I heavy chain,  $\beta_2$ -microglobulin, processing enzymes such as proteases, accessory molecules, chaperones and transporter  
20 proteins such as PSF1.

Within one aspect of the present invention, vector constructs are provided which direct the expression of a protein or active portion of a protein capable of inhibiting MHC antigen presentation. Within the present invention, an "active portion" of a protein is that fragment of the protein which must be retained for biological  
25 activity. Such fragments or active domains can be readily identified by systematically removing nucleotide sequences from the protein sequence, transforming target cells with the resulting recombinant vector construct, and determining MHC class I presentation on the surface of cells using FACS analysis or other immunological assays, such as a CTL assay. These fragments are particularly useful when the size of the sequence  
30 encoding the entire protein exceeds the capacity of the viral carrier. Alternatively, the active domain of the MHC antigen presentation inhibitor protein can be enzymatically digested and the active portion purified by biochemical methods. For example, a monoclonal antibody that blocks the active portion of the protein can be used to isolate and purify the active portion of the cleaved protein. (Harlow et al., Antibodies: A  
35 Laboratory Manual, Cold Springs Harbor, 1988).

Within one embodiment, the recombinant vector construct directs the expression of a protein or active portion of a protein that binds to newly synthesized MHC class I molecules intracellularly. This binding prevents migration of the MHC class I molecule from the endoplasmic reticulum, resulting in the inhibition of terminal glycosylation. This blocks transport of these molecules to the cell surface and prevents cell recognition and lysis by CTL. For instance, one of the products of the E3 gene may be used to inhibit transport of MHC class I molecules to the surface of the transformed cell. More specifically, E3 encodes a 19kD transmembrane glycoprotein, E3/19K, transcribed from the E3 region of the adenovirus 2 genome. Within the context of the present invention, an animal is injected directly with a recombinant vector construct containing the E3/19K sequence, which upon expression produces the E3/19K protein. The E3/19K protein inhibits the surface expression of an MHC class I surface molecules, and the cells transformed by the vector construct evade an immune response. The construction of a representative recombinant vector construct in this regard is presented in Example 7.

Within another embodiment of the present invention, the recombinant vector construct directs the expression of a protein or an active portion of a protein capable of binding  $\beta_2$ -microglobulin. Transport of MHC class I molecules to the cell surface for antigen presentation requires association with  $\beta_2$ -microglobulin. Thus, proteins that bind  $\beta_2$ -microglobulin and inhibit its association with MHC class I indirectly inhibit MHC class I antigen presentation. Suitable proteins include the H301 gene product. Briefly, the H301 gene, obtained from the human cytomegalovirus (CMV) encodes a glycoprotein with sequence homology to the  $\beta_2$ -microglobulin binding site on the heavy chain of the MHC class I molecule (Browne et al., *Nature* 347:770, 1990). H301 binds to  $\beta_2$ -microglobulin, preventing the maturation of MHC class I molecules, and renders transformed cells unrecognizable by cytotoxic T-cells, thus evading MHC class I restricted immune surveillance.

Other proteins, not discussed above, that function to inhibit or down-regulate MHC class I antigen presentation may also be identified and utilized within the context of the present invention. In order to identify such proteins, in particular those derived from mammalian pathogens (and, in turn, active portions thereof), a recombinant vector construct that expresses a protein or an active portion thereof suspected of being capable of inhibiting MHC class I antigen presentation is transformed into a tester cell line, such as BC. The tester cell lines with and without the sequence encoding the candidate protein are compared to stimulators and/or targets in the CTL assay. A

decrease in cell lysis corresponding to the transformed tester cell indicates that the candidate protein is capable of inhibiting MHC presentation.

5 An alternative method to determine down-regulation of MHC class I surface expression is by FACS analysis. More specifically, cell lines are transformed with a recombinant vector construct encoding the candidate protein. After drug selection and expansion, the cells are analyzed by FACS for MHC class I expression and compared to that of non-transformed cells. A decrease in cell surface expression of MHC class I indicates that the candidate protein is capable of inhibiting MHC presentation (see, for instance, Example 12).

10 Within another aspect of the present invention, methods are provided for suppressing an autoimmune response within an animal by transforming tissue cells of an animal with a recombinant vector construct which transcribes an antisense message capable of inhibiting MHC class I antigen presentation. Briefly, oligonucleotides with nucleotide sequences complementary to the protein coding or "sense" sequence are  
15 termed "antisense". Antisense RNA sequences function as regulators of gene expression by hybridizing to complementary mRNA sequences and arresting translation (Mizuno et al., PNAS 81:1966, 1984; Heywood et al., Nucleic Acids Res. 14:6771, 1986). Antisense molecules comprising the entire sequence of the target transcript or any part thereof can be synthesized (Ferretti et al., PNAS 83:599, 1986), placed into vector  
20 constructs, and effectively introduced into cells to inhibit gene expression (Izant et al., Cell 36:1007, 1984). In addition, the synthesis of antisense RNA (asRNA) from DNA cloned in inverted orientation offers stability over time while constitutive asRNA expression does not interfere with normal cell function.

Within one embodiment of the present invention, the recombinant vector  
25 construct transcribes an antisense message capable of binding a conserved region of the MHC class I transcript, thereby inhibiting cell surface expression and MHC class I antigen presentation. One may identify such conserved regions through computer-assisted comparison of sequences representing different classes of MHC genes (for example, HLA A, B and C), available within DNA sequence databanks (e.g., Genbank).  
30 Conserved sequences are identified through computer-assisted alignment for homology of the nucleotide sequences. The conserved region is a sequence having less than 50% mismatch, preferably less than 20% mismatch, per 100 base pairs between MHC class I genotypes.

Within another embodiment of the present invention, the recombinant  
35 vector construct transcribes an antisense message responsible for binding to  $\beta_2$ -microglobulin transcript. This binding prevents translation of the  $\beta_2$ -microglobulin

protein and thereby inhibits proper assembly of the MHC class I molecule complex necessary for cell surface expression. Within a preferred embodiment, the nucleotide sequence for  $\beta_2$ -microglobulin is cloned into a vector construct in the reverse orientation. The proper antisense orientation may be determined by restriction enzyme analysis.

Within still another embodiment, the recombinant vector construct transcribes an antisense message responsible for binding PSF1 transcript, a peptide transporter protein. Since this protein is necessary for the efficient assembly of MHC class I molecules, an antisense to PSF1 transcript blocks the transport of processed antigenic peptide fragments to the endoplasmic reticulum (ER) prior to association with the  $\beta_2$ -microglobulin and MHC class I molecular complex. Within a preferred embodiment, the nucleotide sequence for PSF1 is prepared and inserted in reverse orientation into the vector construct and determined by restriction enzyme analysis.

As discussed above, the sequences of other proteins involved in antigen presentation may also be identified, and used to design a recombinant vector construct capable of transcribing an antisense message that inhibits MHC antigen presentation. More specifically, the nucleotide sequence of the gene encoding the protein is examined, and the identified sequence is used to synthesize an appropriate antisense message. It is preferable to use a sequence complimentary to a portion upstream or close to the start sequence of the target message. This allows the antisense sequence to bind to the mRNA preventing translation of a significant portion of the protein. Examples of such molecules are ICAM-1, ICAM-2, LFA-1, LFA-3, and B7/BB1. Down-regulation of MHC class I expression or antigen presentation may be assayed by FACS analysis or CTL assay, respectively, as described in Examples 13 and 15 or other means as described above for proteins capable of inhibiting MHC class I presentation.

Within another aspect of the present invention, a method is provided for suppressing an autoimmune response within an animal by transducing selected cells of the animal with a recombinant vector construct which transcribes a ribozyme responsible for the enzymatic cleavage of a component involved in MHC antigen presentation. Briefly, ribozymes are RNA molecules with enzymatic activity used to digest other RNA molecules. They consist of short RNA molecules possessing highly conserved sequence-specific cleavage domains flanked by regions which allow accurate positioning of the enzyme relative to the potential cleavage site in the desired target molecule. They provide highly flexible tools in inhibiting the expression and activation of specific genes (Haseloff et al., *Nature* 334:585, 1988). Custom ribozymes can be designed, provided that the transcribed sequences of the gene are known. Specifically, a ribozyme may be



designed by first choosing the particular target RNA sequence and attaching complimentary sequences to the beginning and end of the ribozyme coding sequence. This ribozyme producing gene unit can then be inserted into a recombinant vector construct and used to transform tissue cells. Upon expression, the target gene is  
5 neutralized by complimentary binding and cleavage, guaranteeing permanent inactivation. In addition, because of their enzymatic activity, ribozymes are capable of destroying more than one target.

Within one embodiment, vector constructs containing specific ribozymes are used to cleave the transcript of a conserved region of the MHC class I heavy chain  
10 molecule in order to inhibit antigen presentation. Within another embodiment of the present invention, the recombinant vector construct transcribes a ribozyme responsible for the enzymatic cleavage of the  $\beta_2$ -microglobulin transcript. Specifically, a ribozyme with flanking regions complimentary to a sequence of the  $\beta_2$ -microglobulin message cleaves the transcript, thereby preventing protein translation and proper assembly of the  
15 MHC class I molecule complex. This inhibits transport of the MHC class I complex to the cell surface, thereby preventing antigen presentation.

Within still another embodiment of the present invention, the recombinant vector construct transcribes a ribozyme responsible for the enzymatic cleavage of the PSF1 transcript, thereby suppressing cell surface expression of MHC class I molecules  
20 and preventing antigen presentation. More specifically, a ribozyme designed with flanking regions complimentary to a sequence of the PSF1 message cleaves the transcript and inhibits transport of peptides to the ER, thereby preventing assembly of the MHC class I complex and antigen presentation.

As discussed above, it will be evident to those skilled in the art that the  
25 sequences of other proteins involved in the antigen presentation pathway may be identified and used to design a recombinant vector construct capable of transcribing a ribozyme that inhibits MHC antigen presentation. Down-regulation of MHC class I expression or antigen presentation may be assayed by FACS analysis or CTL assay as described in more detail in Examples 13 and 15 or other means as described above for  
30 proteins and antisense messages capable of inhibiting MHC class I presentation.

Within another aspect of the invention, multivalent recombinant vector constructs are provided. Briefly, the efficiency of suppressing an autoimmune response can be enhanced by transforming cells with a multivalent recombinant vector construct. Upon expression, the gene products increase the degree of interference with MHC  
35 antigen presentation by attacking a single component via two different routes, or through two different components via the same or different route. The construction of

multivalent recombinant vector constructs may require two promoters because one promoter may not ensure adequate levels of gene expression of the second gene. As noted above, a second promoter, such as an internal ribozyme binding site (IRBS) promoter, or herpes simplex virus thymidine kinase (HSVTK) promoter placed in conjunction with the second gene of interest boosts the levels of gene expression of the second gene.

Within preferred embodiments, the vector construct expresses or transcribes at least two of the following components in any combination: (a) a protein or active portion of the proteins E3/19K or H301; (b) an antisense message that binds the transcript of a conserved region of the MHC class I heavy chain,  $\beta_2$ -microglobulin or PSF1 transporter protein; and (c) a ribozyme that cleaves the transcript of the proteins listed in (b) above. In addition, multivalent recombinant vector constructs are provided which express two proteins or active portions of proteins as described herein, two antisense messages, or two ribozymes.

Within related embodiments, a number of specific combinations may be utilized to form a multivalent recombinant vector construct. For example, a multivalent recombinant vector construct may consist of a gene expressing E3/19K or H301 in combination with the antisense message or ribozyme sequence for a conserved region of the MHC class I heavy chain,  $\beta_2$ -microglobulin, or PSF1 transporter protein.

Within another aspect of the present invention, pharmaceutical compositions are provided comprising one of the above described recombinant vector constructs or a recombinant virus carrying the vector construct, such as a retrovirus, poliovirus, rhinovirus, vaccinia virus, influenza virus, adenovirus, adeno-associated virus, herpes simplex virus, measles virus, coronavirus or Sindbis virus, in combination with a pharmaceutically acceptable carrier or diluent. The composition may be prepared either as a liquid solution, or as a solid form (e.g., lyophilized) which is resuspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for either injection, oral, nasal or rectal administration or other means appropriate to the carrier. Generally, the recombinant virus carrying the vector construct is purified to a concentration ranging from 0.25% to 25%, and preferably about 5% to 20% before formulation. Subsequently, after preparation of the composition, the recombinant virus carrying the vector construct will constitute about 10 ng to 1  $\mu$ g of material per dose, with about 10 times this amount of material present as copurified contaminants. Preferably, the composition is prepared in 0.1-1.0 ml of aqueous solution formulated as described below.

Pharmaceutically acceptable carriers or diluents are those which are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline) and containing one or more of mannitol, lactose, trehalose, dextrose, glycerol and ethanol, as well as polypeptides or proteins such as human serum albumin (HSA). One suitable composition comprises a recombinant virus carrying a vector construct in 10 mg/ml mannitol, 1 mg/ml HSA, 20mM Tris pH=7.2 and 150mM NaCl. In this case, since the recombinant virus carrying the vector construct represents approximately 10 ng to 1 µg of material, it may be less than 1% of the total high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is generally stable at -70°C for at least six months. It will be evident that substantially equivalent dosages of the recombinant vector construct may be prepared. In this regard, the vector construct will constitute 100 ng to 100 ug of material per dose, with about 10 times this amount of material present as copurified contaminants.

The composition may be administered through a variety of routes (as discussed above), including intravenous (i.v.), subcutaneous (s.c.), or intramuscular (i.m.) injection. In this regard, it will be evident that the mode of administration will be influenced by the specific therapeutic application. For recombinant viruses carrying the vector construct, the individual doses normally used are  $10^6$  to  $10^{10}$  c.f.u. (e.g., colony forming units of neomycin resistance titered on HT1080 cells). These compositions are administered at one- to four-week intervals for three or four doses (at least initially). Subsequent booster shots may be given as one or two doses after 6-12 months, and thereafter annually.

The following examples are offered by way of illustration and not by way of limitation.

## EXAMPLES

### Example 1

#### PREPARATION OF MURINE RETROVIRAL PROVECTOR DNA

##### 5 A PREPARATION OF RETROVIRAL BACKBONE KT-3B

The Moloney murine leukemia virus (MoMLV) 5' long terminal repeat (LTR) EcoR I-EcoR I fragment, including *gag* sequences, from N2 vector (Armentano et al., *J. Vir.* 61:1647, 1987, Eglitis et al., *Science* 230:1395, 1985) in pUC31 plasmid is ligated  
10 into the plasmid SK<sup>+</sup> (Stratagene, San Diego, CA). The resulting construct is called N2R5. The N2R5 construct is mutated by site-directed *in vitro* mutagenesis to change the ATG start codon to ATT preventing *gag* expression. This mutagenized fragment is 200 base pairs (bp) in length and flanked by Pst I restriction sites. The Pst I-Pst I mutated fragment is purified from the SK<sup>+</sup> plasmid and inserted into the Pst I site of N2  
15 MoMLV 5' LTR in plasmid pUC31 to replace the non-mutated 200 bp fragment. The plasmid pUC31 is derived from pUC19 (Stratagene, San Diego, CA) in which additional restriction sites Xho I, Bgl II, BssH II and Nco I are inserted between the EcoR I and Sac I sites of the polylinker. This construct is called pUC31/N2R5g<sup>M</sup>.

The 1.0 kilobase (Kb) MoMLV 3' LTR EcoR I-EcoR I fragment from N2 was  
20 cloned into plasmid SK<sup>+</sup> resulting in a construct called N2R3<sup>-</sup>. A 1.0 Kb Cla I-Hind III fragment is purified from this construct.

The Cla I-Cla I dominant selectable marker gene fragment from pAFVXM retroviral vector (Kriegler et al., *Cell* 38:483, 1984, St. Louis et al., *PNAS* 85:3150, 1988), comprising a SV40 early promoter driving expression of the neomycin  
25 phosphotransferase gene, is cloned into plasmid SK<sup>+</sup>. A 1.3 Kb Cla I-BstB I gene fragment is purified from the SK<sup>+</sup> plasmid.

An alternative selectable marker, phleomycin resistance (Mulsant et al., *Som. Cell and Mol. Gen.* 14: 243, 1988, available from Cayla, Cedex, FR) may be used to make the retroviral backbone KT-3C, for use in transforming genes to cells that are  
30 already neomycin resistant. The plasmid pUT507 (Mulsant et al., *Som. Cell and Mol. Gen.* 14:243, 1988) is digested with Nde I and the ends blunted with Vlenow polymerase I. The sample is then further digested with Hpa I, Cla I linkers ligated to the mix of fragments and the sample further digested with Cla I. The excess Cla I linkers are removed by Cla I digestion and the 1.2 Kb Cla I fragment carrying the RSV LTR and  
35 the phleomycin resistance gene isolated by agarose gel electrophoresis followed by

purification using Gene Clean (Bio101, San Diego, CA). This fragment is used in place of the 1.3Kb Cla I - Bs+B I neomycin resistance fragment to give the backbone KT-3C.

A further modification of the selectable marker cassette is to simply use the ClaI-Cla I SV2 Neo fragment from PAFVXM. Antisense or Ribozyme sequences can be  
5 inserted into the HWC II S/TE in the 3'-untranslated region of the NEO gene. This vector is designated KT3D.

The expression vector is constructed by a three part ligation in which the Xho I-Cla I fragment containing the gene of interest and the 1.0 Kb MoMLV 3' LTR Cla I-Hind III fragment are inserted into the Xho I-Hind III site of pUC31/N2R5g<sup>M</sup> plasmid.  
10 The 1.3 Kb Cla I-BstB I Neo<sup>r</sup> gene, or 1.2 Kb ClaI PHLEOMYCIN, fragment is then inserted into the Cla I site of this plasmid in the sense orientation.

### Example 2

#### 15 A. CLONING OF E3/19K GENE INTO KT-3B

##### i. ISOLATION AND PURIFICATION OF ADENOVIRUS

The isolation and purification of adenovirus is described by Green et al.,  
20 Methods in Enzymology 58: 425, 1979. Specifically, five liters of Hela cells (3-6 x 10<sup>5</sup> cells/ml) are infected with 100-500 plaque forming units (pfu) per ml of adenovirus type 2 (Ad2) virions (ATCC VR-846). After incubation at 37°C for 30-40 hours, the cells are placed on ice, harvested by centrifugation at 230g for 20 minutes at 4°C, and resuspended in Tris-HCl buffer (pH 8.1). The pellets are mechanically disrupted by  
25 sonication and homogenized in trichlorotrifluoroethane prior to centrifugation at 1,000g for 10 min. The upper aqueous layer is removed and layered over 10 mls of CsCl (1.43 g/cm<sup>3</sup>) and centrifuged in a SW27 rotor for 1 hour at 20,000 rpm. The opalescent viral band is removed and adjusted to 1.34 g/cm<sup>3</sup> with CsCl and further centrifuged in a Ti 50 rotor for 16-20 hours at 30,000 rpm. The visible viral band in the middle of the gradient  
30 is removed and stored at 4°C until purification of adenoviral DNA.

##### ii. ISOLATION AND PURIFICATION OF ADENOVIRUS DNA

The adenovirus band is incubated with protease for 1 hour at 37°C to digest  
35 proteins. After centrifugation at 7,800g for 10 minutes at 4°C, the particles are solubilized in 5% sodium dodecyl sulfate (SDS) at room temperature for 30 minutes

before being extracted with equal volume of phenol. The upper aqueous phase is removed, re-extracted with phenol, extracted three times with ether, and dialyzed in Tris buffer for 24 hours. The viral Ad2 DNA is precipitated in ethanol, washed in ethanol, and resuspended in Tris-EDTA buffer, pH 8.1. Approximately 0.5 mg of viral Ad2 DNA is isolated from virus produced in 1.0 liter of cells.

### iii. ISOLATION OF E3/19K GENE

The viral Ad2 DNA is digested with EcoR I (New England Biolabs, Beverly, MA) and separated by electrophoresis on a 1% agarose gel. The resulting 2.7 Kb Ad2 EcoR I D fragments, located in the Ad2 coordinate region 75.9 to 83.4, containing the E3/19K gene (Herrisse et al., Nucleic Acids Research 8:2173, 1980, Cladaras et al., Virology 140:28, 1985) are eluted by electrophoresis, phenol extracted, ethanol precipitated, and dissolved in Tris-EDTA (pH 8.1).

### iv. CLONING OF E3/19K GENE INTO KT-3B

The E3/19K gene is cloned into the EcoR I site of PUC1813. PUC1813 is prepared as essentially described by Kay et al., Nucleic Acids Research 15:2778, 1987 and Gray et al., PNAS 80:5842, 1983). The E3/19K is retrieved by EcoR I digestion and the isolated fragment is cloned into the EcoR I site of phosphatase-treated pSP73 plasmid, (Promega, Madison, WI). This construct is designated SP-E3/19K. The orientation of the SP-E3/19K cDNA is verified by using appropriate restriction enzyme digestion and DNA sequencing. In the sense orientation, the 5' end of the cDNA is adjacent to the Xho I site of the pSP73 polylinker and the 3' end adjacent to the Cla I site. The Xho I-Cla I fragment containing the E3/19K cDNA in either sense or antisense orientation is retrieved from the SP-E3/19K construct and cloned into the Xho I-Cla I site of the KT-3B retroviral backbone. This construct is designated KT-3B/E3/19K.

### B. CLONING OF PCR AMPLIFIED E3/19K GENE INTO KT-3B

#### i. PCR AMPLIFICATION OF E3/19K GENE

The Ad2 DNA E3/19K gene, including the amino terminal signal sequence, followed by the intraluminal domain and carboxy terminal cytoplasmic tail which allow the E3/19K protein to embed itself in the endoplasmic reticulum (ER), is located

between viral nucleotides 28,812 and 29,288. Isolation of the Ad2 E3/19K gene from the viral genomic DNA is accomplished by PCR amplification, with the primer pair shown below:

- 5 The forward primer corresponds to the Ad2 nucleotide sequences 28,812 to 28,835.

(Sequence ID No. \_\_\_\_\_)

5'-3': TATATCTCCAGATGAGGTACATGATTTTAGGCTTG

The reverse primer corresponds to the Ad2 nucleotide sequences 29,241 to 29,213.

- 10 (Sequence ID No. \_\_\_\_\_)

5'-3': TATATATCGATTCAAGGCATTTTCTTTTCATCAATAAAAC

- 15 In addition to the Ad2 complementary sequences, both primers contain a five nucleotide "buffer sequence" at their 5' ends for efficient enzyme digestion of the PCT amplicon products. This sequence in the forward primer is followed by the Xho I recognition site and by the Cla I recognition site in the reverse primer. Thus, in the 5' to 3' direction, the E3/19K gene is flanked by Xho I and Cla I recognition sites. Amplification of the E3/19K gene from Ad2 DNA is accomplished with the following PCR cycle protocol:

20

Temperature °C	Time (min)	No. Cycles
94	2	1
94	0.5	
55	0.17	5
72	3.5	
94	0.5	30
70	3.5	
72	10	10

ii. LIGATION OF PCR AMPLIFIED E3/19K GENE INTO KT-3B

- 25 The E3/19K gene from the SK-E3/19K construct, approximately 780 bp in length, is removed and isolated by 1% agarose/TBE gel electrophoresis as described in Example 2Bi. The Xho I-Cla I E3/19K fragment is then ligated into the KT-3B

retroviral backbone. This construct is designated KT-3B/E3/19K . It is amplified by transforming DH5 $\alpha$  bacterial strain with the KT-3B/E3/19K construct. Specifically, the bacteria is transformed with 1-1000 ng of ligation reaction mixture DNA. The transformed bacterial cells are plated on LB plates containing ampicillin. The plates are  
5 incubated overnight at 37°C, bacterial colonies are selected and DNA is prepared from them. The DNA is digested with Xho I and Cla I. The expected endonuclease restriction cleavage fragment sizes for plasmids containing the E3/19K gene are 780 and 1300 bp.

10 C. CLONING OF SYNTHESIZED E3/19K GENE INTO KT-3B

i. SYNTHESIS OF E3/19K GENE DNA

Chemical synthesis of synthetic DNA has been previously described (Caruthers et al., Methods in Enzymology 211:3, 1992). Sequences which encode the E3/19K gene  
15 are synthesized by the phosphotriester method on an Applied Biosystems Inc. DNA synthesizer, model 392 (Foster City, CA) using the PCR primer as the 5' and 3' limits and keeping the same Xho I and Cla I linkers on the ends. Short oligonucleotides of approximately 14-40 nucleotides in length are purified by polyacrylamide gel  
20 electrophoresis and ligated together to form the single-stranded DNA molecule (Ferretti et al., PNAS 83:599, 1986) .

ii. SEQUENCING OF E3/19K GENE DNA

25 Fragments are cloned into the bacteriophage vectors M13mp18, and M13mp19 (GIBCO, Gaithersburg, MD), for amplification of the DNA. The nucleotide sequence of each fragment is determined by the dideoxy method using the single-stranded M13mp18 and M13mp19 recombinant phage DNA as templates and selected synthetic oligonucleotides as primers. This confirms the identity and structural integrity of the  
30 gene.

iii LIGATION OF E3/19K GENE INTO KT-3B

The E3/19K gene is ligated into the KT-3B or KT-3C vector as previously  
35 described in Example 2Bii.



Example 3**CLONING OF AN ANTISENSE SEQUENCE OF A CONSERVED REGION OF  
MHC INTO KT-3**

5

**A. CONSTRUCTION OF KT-3Cneo $\alpha$ MHC**

The cDNA clone of the MHC class I allele CW3 (Zemmour et al., Tissue Antigens 39:249, 1992) is used as a template in a PCR reaction for the amplification of  
10 specific sequences to be inserted into the untranslated region of the neomycin resistance gene of the KT-3C backbone vector, .

The MHC class I allele CW1 cDNA is amplified between nucleotide sequence 147 to 1,075 using the following primer pairs:

15 The forward primer corresponds to MHC CW3 cDNA nucleotide sequence 147 to 166:  
(Sequence ID No. \_\_\_\_)

5'-3': TATATGTCGACGGGCTACGTGGACGACACGC

The reverse primer corresponds to MHC CW3 cDNA nucleotide sequence 1,075 to  
20 1,056:

(Sequence ID No. \_\_\_\_)

5'-3': TATATGTCGACCATCAGAGCCCTGGGCACTG

In addition to the MHC class I allele CW3 complementary sequences, both  
25 primers contain a five nucleotide "buffer sequence" at their 5' ends for efficient enzyme digestion of the PCR amplicon products. The buffer sequence is followed by the Hinc II recognition sequence in both primers. Generation of the MHC amplicon with the primers shown above is accomplished using the PCR protocol described in section 2Bi.. This protocol is modified by using Vent polymerase (New England Biolabs, Beverly,  
30 MA) and further modified to include 1 minute extension times instead of 3.5 minutes. The Vent polymerase generates amplicons with blunt ends. Alternatively, the forward and reverse primers may contain only the MHC CW3 complementary sequences.

The MHC CW3 cDNA 950 bp amplicon product is purified with Gene Clean  
(Bio101, San Diego, CA) and digested with Hinc II. The digested fragment, 938 bp, is  
35 isolated by 1% agarose/TBE gel electrophoresis and purified with Gene Clean.

The MHC CW3 cDNA 938 bp fragment is inserted in the 3' untranslated region of the neomycin resistance gene in the antisense orientation. Specifically, the Hinc II recognition sequence at nucleotide sequence number 676 of the pBluescript II SK<sup>+</sup> (pSK<sup>+</sup>) (Stratagene, San Diego, CA) plasmid is removed by digestion with Hinc II and Kpn I. The Kpn I 3' end is blunted with T4 DNA polymerase and the blunt ends are ligated. This plasmid is designated as pSKdIHII. As described in Example 1A, the 1.3 Kb Cla I- Cla I dominant selectable marker gene fragment from pAFVXM retroviral vector is cloned into the Cla I site of pSKdIHII. This plasmid is designated as pSKdIHII/SVneo. The MHC CW3 cDNA 938 bp fragment is inserted in an antisense orientation into the Hinc II site of pSKdIHII/SVneo, located in the 3' untranslated region of the neomycin resistance gene. Confirmation that the MHC CW1 cDNA 938 bp fragment is present in the neomycin gene in an antisense orientation is determined by restriction endonuclease digestion and sequence analysis. This clone is designated as pSKdIHII/SVneo/ $\alpha$ MHC.

Construction of KT-3D/SVneo/ $\alpha$ MHC is accomplished by a three way ligation, in which the Cla I 2.2 Kb SVneo/ $\alpha$ MHC fragment, and the 1.0 Kb MoMLV 3' LTR Cla I-Hind III fragment from N2R3<sup>-</sup>, are inserted between the Cla I and Hind III sites of pUC31/N2R5g<sup>M</sup> plasmid as described in Example 1.

#### B. CONSTRUCTION OF KT-3C/SVneo/VARNA/ $\alpha$ MHC

High level MHC CW3 antisense RNA expression is accomplished by insertion of this sequence downstream of the Ad2 VARNA1 promoter. The Ad2 VARNA promoter-MHC antisense cDNA is assembled as a RNA polymerase III (pol III) expression cassette then inserted into the KT-3C backbone. In this pol III expression cassette, the Ad2 VARNA1 promoter is followed by the antisense  $\alpha$ MHC cDNA, which in turn is followed by the pol III consensus termination signal.

The double stranded -30/+70 Ad2 VARNA1 promoter is chemically synthesized (Railey et al., Mol. Cell. Biol. 8:1147, 1988) and includes Xho I and Bgl II sites at the 5' and 3', respectively.

The VARNA1 promoter forward strand:

(Sequence ID No. \_\_\_\_)

5'-3': CGAGTCTAGACCGTGCAAAAGGAGAGCCTGTAAGCGGGCACTCTTCC  
GTGGTCTGGTGGATAAATTCGCAAGGGTATCATGGCGGACGACCGGGGT  
CGAACCCCGGA

The VARNA1 promoter reverse strand:  
(Sequence ID No. \_\_\_\_)

5'-3': GATCTCCGGGGTTCGAACCCCGGTCGTCGCCATGATACCCTTGCGAA  
TTTATCCACCAGACCACGGAAGAGTGCCCGCTTACAGGCTCTCCTTTTGCA  
CGGTCTAGAC

5

In order to form the double stranded VARNA1 promoter with Xho I and Bgl II cohesive ends, equal amounts of the single strands are mixed together in 10 mM MgCl<sub>2</sub>, heated at 95°C for 5 min then cooled slowly to room temperature to allow the strands to anneal.

10 The MHC class I allele CW3 fragment, nucleotide sequence 653 to 854, from the plasmid pSKd1HII/SVneo/αMHC is amplified using the following primer pair:

The forward primer corresponds to nucleotide sequence 653 to 680:

15 5'-3': TATATCCTAGGTCTCTGACCATGAGGCCACCCTGAGGTG

The reverse primer corresponds to nucleotide sequence 854 to 827:

5'-3': TATATAGATCTACATGGCACGTGTATCTCTGCTCTTCTC

20

In addition to the MHC class I allele CW3 complementary sequences, both primers contain a five nucleotide "buffer sequence" at their 5' ends for efficient enzyme digestion of the PCR amplicon products. The buffer sequence is followed by the Avr II recognition sequence in the forward primer and by the Bgl II recognition sequence in the reverse primer, which allows insertion in an antisense orientation, relative to the Ad2 VARNA1 promoter in the pol III expression cassette. Generation of the MHC amplicon with the primers discussed above is accomplished with the PCR protocol described in Example 2Bi modified to include 0.5 minute extension times instead of 3.5 minutes.

25 The MHC CW3 cDNA 223 bp amplicon product is purified with Gene Clean  
30 (Bio101, San Diego, CA), then digested with AvrII and BglII, and isolated by 2%

NuSeive-1% agarose/TBE gel electrophoresis. The 211 bp band is then excised from the gel and the DNA purified with Gene Clean.

The double stranded pol III consensus termination sequence is chemically synthesized (Geiduschek et al., Annu. Rev. Biochem. 57:873, 1988) and includes Avr II  
5 and Cla I sites at the 5' and 3' ends, respectively.

The pol III termination sequence forward primer:

(Sequence ID No. \_\_\_\_)

5'-3': CTAGGGCGCTTTTTCGCGCAT

10

The pol III termination sequence reverse primer:

(Sequence ID No. \_\_\_\_)

5'-3': CGATGCGCAAAAAGCGCC

15 In order to form the double stranded pol III transcription termination sequence with Avr II and Cla I cohesive ends, equal amounts of the single strands are mixed together in 10 mM MgCl<sub>2</sub>, heated at 95°C for 5 min then cooled slowly to room temperature to allow the strands to anneal.

The pol III expression cassette for antisense  $\alpha$ MHC class I allele CW3 is  
20 assembled in a four way ligation in which the Xho I-Bgl II Ad2 VARNA1 promoter fragment, the Bgl II-Avr II  $\alpha$ MHC CW3 fragment, and the Avr II-Cla I transcription termination fragment, are cloned into pSKII<sup>+</sup> between the Xho I and Cla I sites. This construct is designated pSK/VARNA/ $\alpha$ MHC.

Construction of KT3B/SVneo/VARNA/ $\alpha$ MHC is accomplished in a two step  
25 ligation. The first step is a three way ligation in which the Xho I-Cla I VARNA/ $\alpha$ MHC fragment and the 1.0 Kb MoMLV 3' LTR Cla I-Hind III fragment from N2R3<sup>-</sup>, are inserted between the Xho I and Hind III sites of pUC31/N2R5g<sup>M</sup> plasmid as described in Example 1. This construct is designated KT3B/VARNA/ $\alpha$ MHC. In the second ligation step the 1.3 Kb Cla I-BstB I SVneo fragment into the Cla I site of  
30 KT3B/VARNA/ $\alpha$ MHC. This construct is designated KT3B/SVneo/VARNA/ $\alpha$ MHC.

Example 4**CLONING A RIBOZYME THAT WILL CLEAVE A CONSERVED REGION OF  
MHC CLASS I HEAVY CHAIN INTO KT-3B**

5

**A. CONSTRUCTION OF pSK/VARNA/MHCHRBZ**

In order to efficiently inhibit expression of MHC class I in transduced cells, a hairpin ribozyme with target specificity for the MHC class I allele is inserted into the  
10 KT3B/SVneo vector. The ribozyme is expressed at high levels from the Ad2 VARNA1 promoter. The MHC hairpin ribozyme (HRBZ) is inserted into the pol III pSK/VARNA/ $\alpha$ MHC expression cassette described in Example 3.

The HRBZ and the MHC class I allele CW3 have the homologous sequence shown below:

15 (Sequence ID No. \_\_\_\_)

5'-3': GATGAGTCTCTCATCG

The HRBZ is designed to cleave after the A residue in the AGTC hairpin substrate motif contained in the target sequence. Following cleavage, the HRBZ is  
20 recycled and able to hybridize to, and cleave, other MHC class I RNA molecule.

Double stranded HRBZ as defined previously (Hampel et al., Nucleic Acids Research 18:299, 1990), containing a four base "tetraloop" 3 and an extended helix 4, with specificity for the MHC class I homologous sequence shown above, is chemically synthesized and includes Bgl II and Avr II sites at the 5' and 3' ends, respectively.

25

The MHC HRBZ sense strand:

(Sequence ID No. \_\_\_\_)

5'-3': GATCTCGATGAGAAGAACATCACCAGAGAAACACACGGACTTCGGT  
CCGTGGTATATTACCTGGTAC

30 The MHC HRBZ antisense strand:

(Sequence ID No. \_\_\_\_)

5'-3': CTAGGTACCAGGTAATATACCACGGACCGAAGTCCGTGTGTTTCTCT  
GGTGATGTTCTTCTCATCGA

In order to form the double stranded MHC class I specific HRBZ with Bgl II and Avr II cohesive ends, equal amounts of the single strands are mixed together in 10 mM MgCl<sub>2</sub>, heated at 95°C for 5 min then cooled slowly to room temperature to allow the  
5 strands to anneal.

The pol III expression cassette for the MHC HRBZ is assembled by ligation of the chemically synthesized double stranded MHC class I specific HRBZ with Bgl II and Avr II cohesive ends into Bgl II and Avr II digested and CIAP treated pSK/VARNA/ $\alpha$ MHC, in which the  $\alpha$ MHC sequence has been gel purified away from  
10 the expression vector. This plasmid is designated pSK/VARNA/MHCHRBZ and contains the Ad2 VARNA1 promoter followed by the MHC HRBZ, which in turn is followed by the pol III consensus termination sequence. The pol III expression components is flanked by Xho I and Cla I recognition sites.

#### 15 B. CONSTRUCTION OF KT3B/SVneo/VARNA/MHCHRBZ

Construction of KT3B/SVneo/VARNA/MHCHRBZ is accomplished in a two step ligation. The first step is a three way ligation in which the Xho I-Cla I VARNA/MHCHRBZ fragment and the 1.0 Kb MoMLV 3' LTR Cla I-Hind III fragment from N2R3<sup>-</sup>, are inserted  
20 between the Xho I and Hind III sites of pUC31/N2R5g<sup>M</sup> plasmid described in Example 1. This construct is designated KT3B/VARNA/MHCHRBZ. In the second step, the 1.3 Kb Cla I-BstB I SVneo fragment is ligated into the Cla I site of KT3B/VARNA/MHCHRBZ. This construct is designated KT3B/SVneo/VARNA/MHCHRBZ.

25

### Example 5

#### CLONING OF PSF1 ANTISENSE cDNA

#### 30 A. CONSTRUCTION OF KT-3C/SVneo/ $\alpha$ PSF1

The cDNA clone of PSF1 (Spies et al., Nature 351: 323, 1991; Spies et al., Nature 348: 744, 1990) is used as a template in a PCR reaction for the amplification of specific sequences to be inserted into the KT-3C backbone vector, into the untranslated  
35 region of the neomycin resistant gene. The PSF1 cDNA is amplified between nucleotide sequence 91 to 1,124 using the following primer pairs:

The forward primer corresponds to nucleotide sequence 91 to 111:

(Sequence ID No. \_\_\_\_)

5'-3': TATATGTCGACGAGCCATGCGGCTCCCTGAC

5 The reverse primer corresponds to nucleotide sequence 1,124 to 1,105:

(Sequence ID No. \_\_\_\_)

5'-3': TATATGTCGACCGAACGGTCTGCAGCCCTCC

10 In addition to the PSF1 complementary sequences, both primers contain a five nucleotide "buffer sequence" at their 5' ends for efficient enzyme digestion of the PCR amplicon products. The buffer sequence is followed by the Hinc II recognition sequence in both primers. Generation of the PSF1 amplicon with the primers discussed above is accomplished with the PCR protocol described in Example 2Bi. This protocol is modified by using Vent polymerase (New England Biolabs, Beverly, MA) and further  
15 modified to include 1 minute extension times instead of 3.5 minutes. The Vent polymerase generates amplicons with blunt ends.

#### B. CONSTRUCTION OF KT3B/SVneo/VARNA/ $\alpha$ PSF1

20 High level PSF1 antisense expression is accomplished by insertion of this sequence downstream of the Ad2 VARNA1 promoter. The Ad2 VARNA promoter-PSF1 antisense cDNA is first assembled as a pol III expression cassette then inserted into the KT-3B backbone. In this pol III expression cassette, the Ad2 VARNA1 promoter is followed by the antisense PSF1 cDNA, which in turn is followed by the  
25 pol III consensus termination signal.

The nucleotide sequence 91 to 309 of the PSF1 cDNA are amplified in a PCR reaction using the following primer pair:

The forward primer corresponds to nucleotide sequence 91 to 111:

30 (Sequence ID No. \_\_\_\_)

5'-3': TATATCCTAGGGAGCCATGCGGCTCCCTGAC

The reverse primer corresponds to nucleotide sequence 309 to 288:

(Sequence ID No. \_\_\_\_)

35 5'-3': TATATAGATCTCAGACAGAGCGGGAGCAGCAG

In addition to the PSF1 complementary sequences, both primers contain a five nucleotide "buffer sequence" at their 5' ends for efficient enzyme digestion of the PCR amplicon products. The buffer sequence is followed by the *Avr* II recognition sequence in the forward primer and by the *Bgl* II recognition sequence in the reverse primer, which allows insertion in an antisense orientation, relative to the Ad2 VARNA1 promoter in the RNA polymerase III expression cassette. Generation of the PSF1 amplicon with the primers described above is accomplished with the PCR protocol described in Example 2Bi modified to include 0.5 minutes extension times instead of 3.5 minutes.

10 The MHC CW3 cDNA 240 bp amplicon product is purified with Gene Clean (Bio101, San Diego, CA), then digested with *Avr* II and *Bgl* II, and isolated by 2% NuSeive-1% agarose/TBE gel electrophoresis. The 211 bp band is then excised from the gel and purified with Gene Clean.

Construction of KT3B/SVneo/VARNA/ $\alpha$ PSF1 is accomplished in two step  
15 ligation. The first step is a three-way ligation in which the *Xho* I-*Cla* I VARNA/ $\alpha$ PSF1 fragment and the 1.0 Kb MoMLV 3' LTR *Cla* I-*Hind* III fragment from N2R3<sup>-</sup>, are inserted between the *Xho* I and *Hind* III sites of pUC31/N2R5g<sup>M</sup> plasmid as described in Example 1. This construct is designated as KT3B/VARNA/ $\alpha$ PSF1. In the second ligation step, the 1.3 kb *Cla* I-*Bst*B I SVneo fragment is ligated into the *Cla* I site of  
20 KT3B/VARNA/ $\alpha$ PSF1. This construct is designated KT3B/SVneo/VARNA/ $\alpha$ PSF1.

### Example 6

#### 25 CLONING A RIBOZYME THAT WILL CLEAVE A CONSERVED REGION OF PSF1 INTO KT-3B

##### A. CONSTRUCTION OF pSK/VARNA/PSF1HRBZ

In order to efficiently inhibit expression of PSF1 in transduced cells, a hairpin  
30 ribozyme with target specificity for the PSF1 RNA is inserted into the KT3B/SVneo vector. The ribozyme is expressed at high levels from the Ad2 VARNA1 promoter. The PSF1 hairpin ribozyme (HRBZ) is inserted into the pol III pSK/VARNA/ $\alpha$ MHC expression cassette described in Example 3. The PSF1 HRBZ-pol III expression cassette is then inserted into the KT3B/SVneo backbone vector.

35 The HRBZ and the PSF1 RNA have the homologous sequence shown below:  
(Sequence ID No. \_\_\_\_)



5'-3': GCTCTGTCTGGCCAC

The HRBZ is designed to cleave after the T residue in the IGTC hairpin substrate motif contained in the target sequence. Following cleavage, the HRBZ is recycled and able to hybridize to, and cleave, other PSF1 RNA molecule.

Double stranded HRBZ as defined previously (Hampel et al., Nucleic Acids Research 18:299, 1990), containing a four base "tetraloop" 3 and an extended helix 4, with specificity for the PSF1 homologous sequence shown above, is chemically synthesized and includes Bgl II and Avr II sites at the 5' and 3' ends, respectively.

The PSF1 HRBZ, sense strand:

(Sequence ID No. \_\_\_\_)

5'-3':

GATCTGTGGCCAGACAGAGCACCAGAGAAACACACGGACTTCGGTCC  
GTGGTATATTACCTGGTAC

The PSF1 HRBZ, antisense strand:

(Sequence ID No. \_\_\_\_)

5'-3':

CTAGGTACCAGGTAATATACCACGGACCGAAGTCCGTGTGTTTCTCT  
GGTGCTCTGTCTGGCCACA

In order to form the double stranded PSF1 specific HRBZ with Bgl II and Avr II cohesive ends, equal amounts of the single strands are mixed together in 10 mM MgCl<sub>2</sub> heated at 95°C for 5 min then cooled slowly to room temperature to allow the strands to anneal.

The pol III expression cassette for the PSF1 HRBZ is assembled by ligation of the chemically synthesized double stranded PSF1 specific HRBZ with Bgl II and Avr II cohesive ends into Bgl II and Avr II digested and CIAP treated pSK/VARNA/ $\alpha$ MHC, in which the  $\alpha$ MHC sequence has been gel purified away from the pol III expression vector. This plasmid is designated pSK/VARNA/PSF1HRBZ and contains the Ad2 VARNA1 promoter followed by the PSF1 HRBZ, which in turn is followed by the pol III consensus termination sequence. The pol III expression component is flanked by Xho I and Cla I recognition sites.

#### B. CONSTRUCTION OF KT3B/SVneo/VARNA/PSF1HRBZ

Construction of KT3B/SVneo/VARNA/MHCHRBZ is accomplished in a two step ligation. The first step is a three way ligation in which the Xho I-Cla I VARNA/PSF1HRBZ fragment and the 1.0 Kb MoMLV 3' LTR Cla I-Hind III fragment from N2R3<sup>-</sup>, are inserted  
5 between the Xho I and Hind III sites of pUC31/N2R5g<sup>M</sup> plasmid as described in Example 1. This construct is designated KT3B/VARNA/PSF1HRBZ. In the second ligation step, the 1.3 Kb Cla I-BstB I SVneo fragment is ligated into the Cla I site of KT3B/VARNA/PSF1HRBZ. This construct is designated KT3B/SVneo/VARNA/PSF1HRBZ.

10

#### Example 7

#### CONSTRUCTION OF THE MULTIVALENT RECOMBINANT RETROVIRAL VECTOR KT3B-E3/19K/ $\alpha$ MHC

15 A variation of the retroviral vector KT3B-E3/19K can also be constructed containing both the E3/19K sequences and anti-sense sequences specific for a conserved region between the three class I MHC alleles A2, CW3 and B27, Example 2 and 3. This vector, known as KT3B-E3/19K/ $\alpha$ MHC, is designed to incorporate the MHC class I anti-sense sequences at the 3' end of the E3/19K sequence which would be expressed as  
20 a chimeric molecule. The retroviral vector, KT3B-E3/19K/ $\alpha$ MHC, can be constructed by ligating a Cla I digested PCR amplified product containing the MHC anti-sense sequences into the Cla I site of the KT3B-E3/19K vector. More specifically, the cDNA clone of the MHC class I allele CW3 (Zemmour et al., *Tissue Antigens* 39:249, 1992) is amplified by PCR between nucleotides 653 and 854 using the following primer pair:

25

The forward primer of  $\alpha$ MHC is:

(Sequence ID No. \_\_\_\_\_)

5'-3': ATTATCGATTCTCTGACCATGAGGCCACCCTGAGGTG

30 The reverse primer of  $\alpha$ MHC is:

(Sequence ID No. \_\_\_\_\_)

5'-3': ATTAATCGATACATGGCACGTGTATCTCTGCTCTTCTC

The primer pairs are flanked by Cla I restriction enzyme sites in order to insert an  
35 amplified Cla I digested product into the partially pre-digested KT-3B-E3/19K vector in

the anti-sense orientation. By placing the Cla I fragment in reverse orientation the vector will express the negative anti-sense strand upon transcription.

### Example 8

5

## TRANSDUCTION OF PACKAGING CELL LINES DA WITH THE RECOMBINANT RETROVIRAL VECTOR KT3B-E3/19K OR OTHER VECTORS

### A. PLASMID DNA TRANSFECTION

10

293 2-3 cells (a cell line derived from 293 cells ATCC No. CRL 1573, WO 92/05266)  $5 \times 10^5$  cells are seeded at approximately 50% confluence on a 6 cm tissue culture dish. The following day, the media is replaced with 4 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA coprecipitation is performed by mixing 10.0  $\mu$ g of KT-3B-E3/19K plasmid and 10.0  $\mu$ g MLP G plasmid with a 2M  $\text{CaCl}_2$  solution, adding a 1x Hepes buffered saline solution, pH 6.9, and incubating for 15 minutes at room temperature. The calcium phosphate-DNA coprecipitate is transferred to the 293 2-3 cells, which are then incubated overnight at 37°C, 5%  $\text{CO}_2$ . The following morning, the cells are rinsed three times in 1x PBS, pH 7.0. Fresh media is added to the cells, followed by overnight incubation at 37°C, 10%  $\text{CO}_2$ . The following day, the media is collected off the cells and passed through a 0.45  $\mu$  filter. This supernatant is used to transduce packaging and tumor cell lines. Transient vector supernatant for other vectors are generated in a similar fashion.

### 25 B. PACKAGING CELL LINE TRANSDUCTION

DA cells (an amphotropic cell line derived from D-17 cells ATCC No. 183, WO 92/05266) are seeded at  $5 \times 10^5$  cells/10 cm dish. Approximately 0.5 ml of the freshly collected 293 2-3 supernatant (or supernatant that has been stored at -70°C) is added to the DA cells. The following day, phleomycin is added to these cells and a drug resistant pool is generated over a period of a week. This pool of cells is dilution cloned to yield a single cell per well of 96 well plates. Twenty-four clones are expanded to 24 well plates, then to 6 well plates, at which time cell supernatants are collected for titering. DA clones are selected for vector production and called DA-E3/19K. Vector supernatants are collected from 10cm confluent plates of DA-E3/19K clones cultured in normal media

containing polybrene or protamine sulfate. Alternatively, vector supernatant can be harvested from bioreactors or roller bottles, processed and purified further before use.

For those vectors without a drug resistance marker or with a marker already in the packaging cell line, selection of stably transduced clones must be performed by dilution cloning the DA transduced cells one to two days after transducing the cells with 293 2-3 generated supernatant. The dilution clones are then screened for the presence of E3/19K expression by using reverse transcription of messenger RNA, followed by amplification of the cDNA message by the polymerase chain reaction, a procedure known as the RT-PCR. A commercial kit is available through Invitrogen Corp. (San Diego, CA). RT-PCR should be performed on clones which have been propagated for at least 10 days and approximately 50 to 100 clones will need to be screened in order to find a reasonable number of stably transformed clones. In order to perform RT-PCR, specific primers will be required for each message to be amplified. Primers designed to amplify a 401 bp product for E3/19K message screening are as follows:

Screening primers for E3/19K are:

(Sequence ID No. \_\_\_\_\_)

5'-3': ATGAGGTACATGATTTTAGGCTTG

(Sequence ID No. \_\_\_\_\_)

5'-3': TCAAGGCATTTTCTTTTCATCAATAAAAC

#### Example 9

#### DETECTION OF REPLICATION COMPETENT RETROVIRUSES

The extended  $S^+L^-$  assay determines whether replication competent, infectious virus is present in the supernatant of the cell line of interest. The assay is based on the empirical observation that infectious retroviruses generate foci on the indicator cell line  $MiCl_1$  (ATCC CCL 64.1). The  $MiCl_1$  cell line is derived from the  $Mv1Lu$  mink cell line (ATCC CCL 64) by transduction with Murine Sarcoma Virus (MSV). It is a non-producer, non-transformed, revertant clone containing a murine sarcoma provirus that forms sarcoma ( $S^+$ ) indicating the presence of the MSV genome but does not cause leukemia ( $L^-$ ) indicating the absence of replication competent virus. Infection of  $MiCl_1$  cells with replication competent retrovirus "activates" the MSV genome to trigger "transformation" which results in foci formation.

Supernatant is removed from the cell line to be tested for presence of replication competent retrovirus and passed through a 0.45  $\mu$  filter to remove any cells. On day 1, Mv1Lu cells are seeded at  $1 \times 10^5$  cells per well (one well per sample to be tested) of a 6 well plate in 2 ml DMEM, 10% FBS and 8  $\mu$ g/ml polybrene. Mv1Lu cells are plated in the same manner for positive and negative controls on separate 6 well plates. The cells are incubated overnight at 37°C, 10% CO<sub>2</sub>. On day 2, 1.0 ml of test supernatant is added to the Mv1Lu cells. The negative control plates are incubated with 1.0 ml of media. The positive control consists of three dilutions (200 focus forming units (ffu), 20 ffu and 2 ffu each in 1.0 ml media) of MA virus (Miller et al., Molec. and Cell. Biol. 5:431, 1985) which is added to the cells in the positive control wells. The cells are incubated overnight. On day 3, the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. The cells are allowed to grow to confluency and are split 1:10 on day 6 and day 10, amplifying any replication competent retrovirus. On day 13, the media on the Mv1Lu cells is aspirated and 2.0 ml DMEM and 10% FBS is added to the cells. In addition, the MiCl<sub>1</sub> cells are seeded at  $1 \times 10^5$  cells per well in 2.0 ml DMEM, 10% FBS and 8  $\mu$ g/ml polybrene. On day 14, the supernatant from the Mv1Lu cells is transferred to the corresponding well of the MiCl<sub>1</sub> cells and incubated overnight at 37°C, 10% CO<sub>2</sub>. On day 15, the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. On day 21, the cells are examined for focus formation (appearing as clustered, refractile cells that overgrow the monolayer and remain attached) on the monolayer of cells. The test article is determined to be contaminated with replication competent retrovirus if foci appear on the MiCl<sub>1</sub> cells.

#### Example 10

#### TRANSDUCTION OF CELL LINES WITH E3/19K RETROVIRAL VECTOR

The following adherent human and murine cell lines are seeded at  $5 \times 10^5$  cells/10 cm dish with 4  $\mu$ g/ml polybrene: HT 1080 (ATCC No. CCL 121), Hela (ATCC No. CCL 2), BC10ME (Patek et al., Cell. Immuno. 72:113, 1982, ATCC No. TIB 85), BCenv, BC10ME expressing HIV-1 IIIEnv (Warner et al., AIDS Res. and Human Retroviruses 7:645, 1991, L33 obtained from Gunther Oennert, University of Southern California), and L33env. The following day, 1.0 ml of filtered supernatant from the DA E3/19K pool is added to each of the cell culture plates. The following day, phleomycin is added to the media of all cell cultures. For cell lines that are already neomycin

resistant, the E3/19K in the KT-3C backbone (phleomycin resistant) is used. Transient supernatants for 293 2-3 or from DA derived lines can be used." The cultures are maintained until selection is complete and sufficient cell numbers are generated to test for gene expression. The transduced cell lines are designated HT 1080-E3/19K, Hela-  
5 E3/19K, BC10ME-E3/19K, L33-E3/19K and L33env-E3/19K respectively.

EBV transformed cell lines (BLCL), and other suspension cell lines, are transduced by co-cultivation with the irradiated producer cell line, DA-E3/19K. Specifically, irradiated (10,000 rads) producer line cells are plated at  $5 \times 10^5$  cells /6 cm dish in growth media containing 4  $\mu$ g/ml polybrene. After the cells have been allowed  
10 to attach for 2-24 hours,  $10^6$  suspension cells are added. After 2-3 days, the suspension cells are removed, pelleted by centrifugation, resuspended in growth media containing 1mg/ml phleomycin, and seeded in 10 wells of a round bottom 96 well plate. The cultures were expanded to 24 well plates, then to T-25 flasks.

15

#### Example 11

### EXPRESSION OF E3/19K IN THE MULTIVALENT RECOMBINANT RETROVIRAL VECTOR CONSTRUCT KT3B-E3/19K

#### 20 A. WESTERN BLOT ANALYSIS FOR E3/19K

Radio-immuno precipitation assay (RIPA) lysates are made from selected cultures for analysis of E3/19K expression. RIPA lysates are prepared from confluent plates of cells. Specifically, the media is first aspirated off the cells. Depending upon the  
25 size of the culture plate containing the cells, a volume of 100 to 500  $\mu$ l ice cold RIPA lysis buffer (10 mM Tris, pH 7.4; 1% Nonidet P40 (Calbiochem, San Diego, CA); 0.1% SDS; 150 mM NaCl) is added to the cells. Cells are removed from plates using a micropipet and the mixture is transferred to a microfuge tube. The tube is centrifuged for 5 minutes to precipitate cellular debris and the supernatant is transferred to another  
30 tube. The supernatants are electrophoresed on a 10% SDS-PAGE gel and the protein bands are transferred to an Immobilon membrane in CAPS buffer (Aldrich, Milwaukee, WI) (10 mM CAPS, pH 11.0; 10% methanol) at 10 to 60 volts for 2 to 18 hours. The membrane is transferred from the CAPS buffer to 5% Blotto (5% nonfat dry milk; 50 mM Tris, pH 7.4; 150 mM NaCl; 0.02% sodium azide, and 0.05% Tween 20) and  
35 probed with a mouse monoclonal antibody to E3/19K (Severinsson et al., J. Cell Biol.

101:540, 1985). Antibody binding to the membrane is detected by the use of  $^{125}\text{I}$ -Protein A.

### Example 12

5

#### FACS ANALYSIS OF E3/19K-VECTOR TRANSDUCED CELLS TO DEMONSTRATE DECREASED LEVELS OF CLASS I EXPRESSION COMPARED TO NON-TRANSDUCED CELLS.

- 10 Cell lines transduced with the E3/19K-vector are examined for MHC class I molecule expression by FACS analysis. Non-transduced cells are also analyzed for MHC class I molecule expression and compared with E3/19K transduced cells to determine the effect of transduction on MHC class I molecule expression.
- 15 Murine cell lines, L33-E3/19K, L33env-E3/19K, L33, L33env, BC10ME, BCEnv, and BCEnv-E3/19K, are tested for expression of the H-2D<sup>d</sup> molecule on the cell surface. Cells grown to subconfluent density are removed from culture dishes by treatment with Versene and washed two times with cold (4°C) PBS plus 1% BSA and 0.02% Na-azide (wash buffer) by centrifugation at 200g. Two million cells are placed in microfuge tubes and pelleted by centrifugation, 200g, and the supernatant is removed.
- 20 Cell pellets are resuspended with the H-2D<sup>d</sup>-specific Mab 34-2-12s (50μl of a 1:100 dilution of purified antibody, ATCC No. HB 87) and incubated for 30 min at 4°C with occasional mixing. Antibody labeled cells are washed two times with 1 ml of wash buffer (4°C) centrifuged and the supernatant is removed. Cells are resuspended with a biotinylated goat anti-mouse kappa light chain Mab (Amersham, Arlington Heights, IL)
- 25 (50μl, of a 1:100 dilution of purified antibody) and incubated for 30 min at 4°C. Cells are washed, resuspended with 50μl of avidin conjugated FITC (Pierce, Rockford, IL), and incubated for 30 min at 4°C. The cells are washed once more, resuspended in 1 ml of wash buffer, and held on ice prior to analysis on a FACStar Analyzer (Becton Dickinson, Los Angeles, CA). The mean fluorescence intensity of transduced cells is
- 30 compared with that of non-transduced cells to determine the effect E3/19K protein has on surface MHC class I molecule expression.

Example 13

## MURINE CTL ASSAY

5 Balb/c mice are injected with  $10^7$  irradiated (10,000 rads) BCenv cells. After 7 days the spleens are harvested, dispersed into single cell suspension and  $3 \times 10^6$  splenocytes/ml are cultured *in vitro* with  $6 \times 10^4$  cells/ml irradiated BCenv or BCenv-E3/19K cells for 7 days at 37°C in T-25 flasks. Culture medium consists of RPMI 1640; 5% fetal bovine serum, heat-inactivated (FBS); 1 mM pyruvate; 50 µg/ml  
10 gentamicin and  $10^{-5}$  M 2-mercaptoethanol. Effector cells are harvested 7 days later and tested using various effector:target cell ratios in 96 well microtiter plates in a standard 4-6 hour assay. The assay employs  $\text{Na}_2^{51}\text{CrO}_4$ -labeled, 100 µCi, 1 hour at 37°C, (Amersham, Arlington Heights, Illinois) target cells (BC, BCenv, Warner, et al., Aids Res. and Human Retroviruses 7: 645, 1991 or BCenv E3/19K) at  $1.0 \times 10^4$  cells/well  
15 with the final total volume per well of 200 µl. Following incubation, 100 µl of culture medium is removed and analyzed in a WALLAC gamma spectrometer (Gaithersburg, MD.). Spontaneous release (SR) is determined as CPM from targets plus medium and maximum release (MR) is determined as counts per minute (CPM) from targets plus 1M HCl. Percent target cell lysis is calculated as: [(effector cell + target CPM) -  
20 (SR)]/[(MR) - (SR)] x 100. Spontaneous release values of targets are typically 10%-30% of the MR. Tumor cells that have been transduced with the gene of interest (ribozyme, E3/19K, antisense, etc.) are used as stimulator and/or target cells in this assay to demonstrate the reduction of HIV-specific CTL induction and detection as compared to the non-transduced line which is the positive control.

25

Example 14

TUMOR REJECTION OF L33ENV CELLS BY BALB/C MICE IS ABROGATED  
WHEN CLASS I MOLECULE SURFACE EXPRESSION IS DECREASED BY THE  
30 E3-VECTOR TRANSDUCTION.

The L33env cell is being employed as a model for gene therapy treated transformed cells. Gene therapy treated cells produce a foreign protein making them possible targets for clearance by CTL. It has been demonstrated that Balb/c mice  
35 injected with live L33 tumor cells will develop a solid tumor identifiable by caliper measurement within three weeks post-exposure. However, Balb/c mice injected with



- live L33env transformed tumor cells (L33 cells transduced and selected for expression of the HIV-1III<sub>B</sub> envelope protein) recognize HIV env in the context of H-2D<sup>d</sup> and reject the tumor cells with no apparent tumor up to 15 weeks later (Warner et al., AIDS Res. and Human Retroviruses 7:645, 1991). Transformation of L33env cells with the
- 5 E3/19K vector decreases cell surface expression of MHC class I molecules allowing these cells to evade immune surveillance and thereby establish a tumor. Development of an L33env tumor indicates that cell surface expression of MHC class I molecules has been decreased by cotransducing cells with the E19 gene. This impedes optimal immune system clearance mechanisms.
- 10 Three tumor cell lines L33, L33env, and E3/19K-L33env are grown in DMEM containing 10% FBS. The tumor cells are gently rinsed with cold (4°C) PBS and treated with versene to remove them from the plate. After aspirating cells from plates, single cell suspensions are added to sterile plastic tubes. Cell suspensions are washed two
- 15 times in sterile PBS (4°C), counted and resuspended in PBS to 10<sup>7</sup> cells/ml. Balb/c mice (4-6 weeks old) are injected subcutaneous with 10<sup>6</sup> live tumor cells (0.1 ml) and assessed for tumor formation and tumor clearance. Different mice are injected with different tumor cell lines. Mice injected with L33 cells are positive control animals for tumor formation while those injected with L33env are negative controls and should reject the tumor cells because of the env specific CTL response. The group of mice
- 20 injected with E3/19K-transformed, L33env cells are monitored to show the effect that E3/19K expression in L33env cells has on the murine immune response to these tumor cells.

#### Example 15

25

#### FACS ANALYSIS OF E3/19K-VECTOR TRANSDUCED HUMAN CELLS TO DEMONSTRATE DECREASED LEVELS OF MHC CLASS I EXPRESSION COMPARED TO NON-TRANSDUCED CELLS.

30

Cell lines transduced with the E3/19K vector are examined for class I molecule expression by FACS analysis. Non-transduced cells are analyzed for class I molecule expression to compare with E3/19K transduced cells and determine the effect that transduction has on class I molecule expression.

35

Two human cell lines, JY-E3/19K and JY (ATCC No. \_\_\_\_ ) are tested for expression of the HLA-A2 molecule on the cell surface. Suspension cells grown to 10<sup>6</sup> cells/ml are removed from culture flasks by pipet and washed two times with cold (4°C)

PBS plus 1% BSA and 0.02% Na-azide (wash buffer) by centrifugation at 200g. Two million ( $2 \times 10^6$ ) cells are placed in microfuge tubes, pelleted in at 200g, and the supernatant is removed. Cell pellets are resuspended with the HLA-A2-specific Mab BB7.2 (50 $\mu$ l of a 1:100 dilution of purified antibody, ATCC No. HB 82) and incubated with antibody for 30 min at 4°C with occasional mixing. Antibody labeled cells are washed two times with 1 ml of wash buffer (4°C). Prior to removing the supernatant, the cells are resuspended with a biotinylated rat anti-mouse kappa light chain Mab (50 $\mu$ l, of a 1:100 dilution of purified antibody) and incubated for 30 min at 4°C. Cells are washed, resuspended with 50 $\mu$ l of avidin conjugated FITC, and incubated for 30 min at 4°C. The cells are washed once more, and resuspended in 1 ml of wash buffer, and held on ice prior to analysis on a FACStar Analyzer. The mean fluorescence intensity of transduced cells is compared with that of non-transduced cells to determine the effect E3/19K protein has on surface MHC class I molecule expression.

15

#### Example 16

#### MEASUREMENT OF THE IMMUNE RESPONSE TO E3/19K-TRANSDUCED AND NONTRANSDUCED EBV-TRANSFORMED HUMAN JY CELLS BY HLA-A2 RESTRICTED, EBV-SPECIFIC HUMAN CTL LINES.

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Human CTL lines propagated from donor blood samples using autologous EBV transformed cells as stimulators have been shown to be HLA-A2 restricted and specific for EBV proteins. These CTL lines, are propagated with autologous EBV transformed cells and can lyse JY target cells (HLA-A2<sup>+</sup> and EBV transformed). A chromium release assay can be performed with these CTL lines and JY target cells that have been transformed with the E3/19K gene or nontransduced. The E3/19K transformed JY target cell are used to demonstrate decreased recognition and lysis of this cell when compared to nontransformed JY target cells. These results indicate that cell transformation with agents that decrease MHC class I surface expression also decreases MHC class I restricted cell mediated immune responses in an *in vitro* human cell model system.

Approximately,  $1 \times 10^6$  irradiated (10,000 rad) JY cells are cultured with  $1 \times 10^7$  PBMC from a person that is HLA-A2 and verified to have an EBV response, in 10 mls of culture medium at 37°C 5% CO<sub>2</sub> for 7-10 days. The culture medium consists of RPMI 1640 supplemented with 5% heat inactivated fetal bovine serum preselected for CTL growth, 1 mM sodium pyruvate and nonessential amino acids. After the 7-10 day

- incubation the effector cells are harvested and tested in a standard 4-6 hour chromium release assay using  $^{51}\text{Cr}$  labeled JY cells as the positive control and  $^{51}\text{Cr}$  labeled JY-E3/19K. JY and JY-E3/19K cells are labeled with 300  $\mu\text{Ci}$  of  $\text{Na}_2^{51}\text{CrO}_4$  for 1 hour at  $37^\circ\text{C}$ , then washed, counted, and used in the assay at  $4 \times 10^3$  cells/well with the final
- 5 total volume per well of 200  $\mu\text{l}$ . Following incubation, 100  $\mu\text{l}$  of culture medium is removed and analyzed in a WALLAC gamma spectrometer. Spontaneous release (SR) is determined as counts per minute (CPM) CPM from targets plus medium and maximum release (MR) is determined as from targets plus 1M HCl. Percent target cell lysis is calculated as:  $[(\text{effector cell} + \text{target CPM}) - (\text{SR})]/[(\text{MR}) - (\text{SR})] \times 100$ .
- 10 Spontaneous release values of targets are typically 10%-30% of the MR. Tumor cells that have been transduced with the gene of interest (ribozyme, E3/19K, antisense, etc.) are used as stimulator and/or target cells in this assay to demonstrate the reduction of EBV-specific CTL induction and detection as compared to the non-transduced line which is the positive control.
- 15 The number of transformed cells used to infuse back into the patient per infusion is projected to be at a minimum of  $10^7$  -  $10^8$  cells per patient per injection. The site of the infusion may be directly into the patient's synovial membrane or i.v., into the peripheral blood stream.
- 20 From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

### Claims

1. Tissue cells of an animal transformed with a recombinant vector construct that expresses a protein or active portion of a protein capable of inhibiting MHC antigen presentation, for use in a method for suppressing an autoimmune response within an animal.

2. The cells of claim 1 wherein the recombinant vector construct directs the expression of a protein capable of binding  $\beta_2$ -microglobulin.

3. The cells of claim 1 wherein the recombinant vector construct directs the expression of a protein capable of binding the MHC class I heavy chain molecule intracellularly.

4. The cells of claim 1 wherein the recombinant vector construct directs the expression of a protein or active portion of a protein selected from the group consisting of E3/19K and H301.

5. Tissue cells of an animal transformed with a recombinant vector construct that transcribes an antisense message capable of inhibiting MHC antigen presentation, such that an autoimmune response against the cells is suppressed, for use in a method of suppressing an autoimmune response within an animal.

6. The cells of claim 5 wherein the recombinant vector construct transcribes an antisense message which binds a conserved region of MHC class I heavy chain transcripts.

7. The cells of claim 5 wherein the recombinant vector construct transcribes an antisense message which binds the  $\beta_2$ -microglobulin transcript.

8. The cells of claim 5 wherein the recombinant vector construct transcribes an antisense message which binds the PSF1 transporter protein transcript.

Tissue cells of an animal transformed with a recombinant vector construct that transcribes a ribozyme capable of inhibiting MHC antigen presentation, such

that an autoimmune response against the cells is suppressed, for use in a method of suppressing an autoimmune response within an animal.

10. The cells of claim 9 wherein the recombinant vector construct transcribes a ribozyme that cleaves a conserved region of MHC class I heavy chain transcripts.

11. The cells of claim 9 wherein the recombinant vector construct transcribes a ribozyme that cleaves the  $\beta_2$ -microglobulin transcript.

12. The cells of claim 9 wherein the recombinant vector construct transcribes a ribozyme that cleaves the PSF1 transporter protein transcript.

13. Tissue cells of an animal transformed with a multivalent recombinant vector construct which directs the expression of a protein or active portion of a protein capable of inhibiting MHC antigen presentation, and an antisense message or ribozyme capable of inhibiting MHC antigen presentation, such that an autoimmune response against the cells is suppressed, for use in a method of suppressing an autoimmune response within an animal.

14. Tissue cells of an animal transformed with a multivalent recombinant vector construct which directs the expression of an antisense message and a ribozyme capable of inhibiting MHC antigen presentation, such that an autoimmune response against the cells is suppressed, for use in a method of suppressing an autoimmune response within an animal.

15. Tissue cells of an animal transformed with a multivalent recombinant vector construct which directs the expression of (a) two or more proteins or active portions of proteins, capable of inhibiting MHC antigen presentation; (b) two or more antisense messages capable of inhibiting MHC antigen presentation; or (c) two or more ribozymes capable of inhibiting MHC antigen presentation, such that an autoimmune response against the cells is suppressed, for use in a method of suppressing an autoimmune response within an animal.

16. The cells of claim 13 or 15 wherein the protein is E3/19K or H301 or an active portion thereof.

17. The cells of any one of claims 13, 14 or 15 wherein the antisense message binds to the transcript of a protein selected from the group consisting of a conserved region of the MHC class I heavy chains,  $\beta_2$ -microglobulin and PSF1 transporter protein.

18. The cells of any one of claims 13, 14 or 15 wherein the ribozyme cleaves the transcript of a protein selected from the group consisting of a conserved region of the MHC class I heavy chains,  $\beta_2$ -microglobulin and PSF1 transporter protein.

19. The cells of any one of claims 1, 5, 9, 13, 14 or 15 wherein said recombinant vector construct is a recombinant viral vector construct.

20. The cells of any one of claims 1, 5, 9, 13, 14 or 15 wherein the recombinant vector construct is a recombinant retroviral vector construct.

21. The cells of any one of claims 1, 5, 9, 13, 14 or 15 wherein the recombinant vector construct is carried by a recombinant virus selected from the group consisting of poliovirus, rhinovirus, vaccinia virus, influenza virus, adenovirus, adeno-associated virus, herpes simplex virus, and measles virus.

22. The cells of any one of claims 1, 5, 9, 13, 14 or 15 wherein the recombinant vector construct is carried by a recombinant virus selected from the group consisting of togaviridae, picornaviridae, poxviridae, adenoviridae, parvoviridae, herpesviridae, paramyxoviridae and coronaviridae viruses.

23. The cells of any one of claims 1, 5, 9, 13, 14 or 15 wherein the recombinant vector construct is carried by a recombinant corona virus.

24. The cells of any one of claims 1, 5, 9, 13, 14 or 15 wherein the recombinant vector construct is carried by a recombinant Sindbis virus.

25. The cells of any one of claims 1, 5, 9, 13, 14 or 15 wherein the tissue cells are selected from the group consisting of synovial membrane cells, pancreatic islet cells, hepatocytes and keratocytes.